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Cancer arises from an accumulation of multiple mutations that may occur in oncogenes, tumor suppressor genes or DNA repair genes. Tumor suppressors control cell cycle and growth and mutations or alterations in these suppressors can be associated with the uncontrolled growth of malignant tumors. In this project, two tumor suppressors are targeted: the well-characterized Rb protein and a new protein that binds to it called RIZ, which is itself, a tumor suppressor. The goal is to use x-ray crystallography to study the interactions between Rb and RIZ and to identify the molecular contacts in these interactions. The results will be important to understanding the role of the new regulator protein RIZ in tumorigenesis in breast cancer. This IDEA project is focused on the first step in the process, i.e., crystallization of the proteins. The results to date report the growth of crystals of one functional domain of RIZ and of the pocket domain of Rb.

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Kathryn R. Ely 5/30/00

PI - Signature Date

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INTRODUCTION

Cancer arises from the accumulation of multiple mutations and these mutations can arise in oncogenes, tumor suppressor genes or DNA repair genes. Alterations in tumor suppressor genes are associated with a variety of cancers. A model proposing that both normal alleles of a tumor suppressor gene must be mutated for loss of function and tumorigenesis (9) has been generally confirmed by the identification of more than a dozen tumor suppressors. In this study, we target two proteins that may function as tumor suppressors in breast cancer. One is the well-studied retinoblastoma (Rb) gene product (3, 5, 14) and the other is a newly identified Rb-binding protein RIZ that is just now being characterized (6, 10, 15).

The PR domain is located in the amino-terminal sequence of RIZ (see Fig. 1). The RIZ gene normally encodes two gene products: the full-length protein (~280 kDa) and a shorter product (~250 kDa) called RIZ1 and RIZ2 respectively. RIZ2 lacks the PR domain but the rest of the sequence is identical to RIZ1 (11). Cloning of RIZ by Dr. Huang led to the identification of a new protein motif (1, 7) found in a family within the Krüppel-like zinc finger genes that includes the MDS1-EVI1 leukemia gene and the PRD-BF1 or BLIMP1 transcriptional repressor involved in B cell maturation (2, 8, 13). The MDS1-EVI1 gene also gives rise to a product lacking PR, the EVI1 oncogene (2). A sequence alignment of the PR domains in several related proteins is presented in Fig. 2. By direct comparison of gene products that bear or lack the PR domain, the suggestion is that proteins containing the PR domain are tumor suppressors. Significantly, when Dr. Huang generated mice lacking RIZ1 using gene targeting technology, the incidence rate for tumors was remarkable, i.e., 67% (8 of 12 mice). Following up on the conclusion from these results that loss of RIZ1 predisposes for tumor formation, RIZ1 expression was tested in human tumor tissues and tissue-derived cell lines (4). The results showed that RIZ1 expression is decreased or lost in human breast cancer, neuroblastoma or lung cancer cells whereas expression of RIZ2 is normal. The fact that underexpression of RIZ1 may be common in human breast tumors is a new observation that may represent an innovative tool for diagnosis or possibly treatment. In this project, we focus on the structure of the PR domain to gain insight into the molecular features of this new protein motif.

BODY

As stated in the original application, the long-term goal of this project is to generate three-dimensional crystal structures of two tumor suppressors and the complex between them. Not all proteins can be crystallized. However, this IDEA project was funded to support an intense effort to produce soluble proteins and complexes of the tumor suppressors and then to crystallize the subject proteins. There is seldom a format to support this stage of the efforts in a crystallographic study. Consequently, some projects fail because funding is not available to support the pilot studies. Here, with funding from USAMRMC, we can devote considerable resources to the effort, to maximize the prospects for success. Success will be measured by production of crystals, and time-lines may vary from original projections when soluble proteins are produced. Progress to date is outlined in the following sections.

TASK 1: To generate and crystallize stable complexes of the "pocket domain" of the retinoblastoma protein bound to the AR E1A-like domain of the Rb-binding protein RIZ (Months 1-24)

Crystals of the Rb "pocket" domain have been obtained in space group *P*622 with a=b=110.2Å, c=154.6Å, ∂=120°, and the crystals diffract to high resolution (2Å). At the time of the submission of the original application, although these crystals were in hand, the crystal structure of another modified fragment of the Rb "pocket" was determined in another laboratory (10). Our goal instead, is to crystallize the Rb "pocket" domain in complex with the AR domain from RIZ.

The AR domain of RIZ is cloned as a fusion protein with glutathione-S-transferase. The Rb protein encompassing the full "pocket" domain is cloned in the pET expression system. The GST-AR fusion protein contains a site, which can be cleaved by thrombin to release the AR domain. The free AR fragment is soluble after release from the GST fusion partner. To begin the structural studies, the purification protocols were scaled up. The affinity purification of the GST-AR domain was not straightforward. Apparently an interfering substance from the bacterial culture is generated when the production is scaled up to the multi-liter level. This "substance" inhibits binding affinity of the GST-AR protein to the glutathione resin and yields of purified fusion protein from the lysate are reduced. Efforts are now underway to eliminate the interfering substance.

TASK 2: To solve the crystal structure of the Rb "pocket" domain/RIZ AR domain complex (Months 12-24)

This part of the project is dependent on the successful completion of the work outlined in Task #1.

TASK 3: To characterize the protein-protein interactions of the PR domain of RIZ (Months 1-14).
The work in this task is linked to the work in Task #4 since both Tasks rely on the successful production of soluble protein.

TASK 4: To crystallize the PR domain of RIZ alone or in complex with the interacting region of RIZ oligomerization (Months 6-24).
As shown in Figure 2, there are now several members of the PR family that have been recognized by our collaborator, Dr. Huang.

The PR domains from four molecules shown in Figure 2 have been cloned as GST-fusion proteins; so far, three have been expressed in large scale bacterial culture and purified by affinity chromatography on glutathione-agarose columns. GST-fusion partners have been released from the PR domains by thrombin digestion. The resulting products are purified by additional ion-exchange steps. To ensure success with this new and uncharacterized motif, several PR domains are candidates for crystallization. The purified domains have been tested for solubility by concentration and evaluated by dynamic light scattering. This method measures the aggregation state of the proteins and can be used as a positive indicator for successful crystallization. Samples that are monodisperse in solution are likely to crystallize.

The best success to date has been with the PR domain of the BLPR protein from PRDI-BF1. The purification of this domain is shown in Figure 3. This domain was entered into crystallization trials. Small crystals formed readily overnight from solutions to which a mixture of jeffamine and ammonium sulfate precipitants had been added. Hundreds of tiny needles form. These needles have been used in seeding trials to attempt to convert the small crystals into single large crystals. During the past six months, the conditions were modified to control the pH of the crystallization drops. With time, single block-shaped or hexagonally shaped crystals grew slowly in the vapor diffusion drops. Technically, these crystals are not optimal since they grow only after several months incubation and the reproducibility of their production is difficult to control. Nevertheless, the small block crystals were tested for diffraction at the Stanford Synchrotron Laboratory (SSRL). Diffraction was not suitable to pursue structure solution.

To address the question of reproducibility and to produce crystals that diffract at SSRL, we have initiated a new approach to improve the protein interactions within the crystal lattice. The predicted secondary structure of BLPR was evaluated and the sequence of the domain was analyzed in a hydrophilicity plot. This analysis, when correlated with secondary structure prediction, can predict regions of the sequence that may be located on the surface of the protein. These analyses were used to identify residues that could be involved in crystal contacts within the crystal lattice. Eight residues were marked by these methods

that may be surface residues and the residues were changed using the Quikchange™ method for site-directed mutagenesis. Residues selected included single cysteines, charged residues that appear in clusters or hydrophobic residues that fall in regions that are otherwise highly hydrophilic. Substitutions of alanine or small hydrophilic residues were made singly at each of the eight sites. The mutant proteins were then cloned as GST-fusion proteins. The mutants each differ from wild-type by one residue. Each of these proteins has been purified by affinity chromatography and now each of the mutant proteins is also a candidate for crystallization. During the next funding period, these mutants will be tested for crystallization using the same conditions that produced the small needles. Or new screens testing expanded conditions will be implemented.

In an alternate approach, the PR domain of PDRF-BF1 has been tested by NMR. This domain is soluble at concentrations nearing 100mg/ml, and thus may represent an excellent candidate for structure solution by NMR. In the first analysis, an ID spectrum was recorded. As can be seen from the spectrum (see Figure 4, there is clear amide dispersion, suggesting that this domain has regular ordered structure. The results of course also indicate that the domain is a suitable candidate for structural analysis by NMR. Although NMR studies were not projected in the original application, this does represent a reasonable alternate approach if larger crystals cannot be produced. If during the coming year we decide to pursue NMR studies, we will seek approval from the Grants Officer. But first, priority will be given to the crystallization trials and the formation of complex.

It is possible that the PR domains will not crystallize alone, but rather will only crystallize when bound to the partner domain in the intact protein. Dr. Huang has made the observation that the PR domain of RIZ is a protein-protein interaction domain. This will be tested with purified proteins. Dr. Huang already has the constructs in hand to evaluate binding of the PR domain to the PR binding segment in the carboxyl segment of RIZ (4). He has shown that a region between residues 1514 and 1680 in RIZ contains the PR binding site and is sufficient for this binding.

Similarly, we will generate the C-terminal protein interaction domain in PDFI-BF1 as it is identified by Dr. Huang. This domain will then be available as a GST-fusion protein and purified on a glutathione affinity column. We will use SPR to monitor the binding of the PR domain to this region of PDFI-BF-1. Immobilization of one fragment will be accomplished either with the standard amine coupling to the dextran chips or we may choose to couple the PR domain to the chip as a GST-fusion protein utilizing commercially available anti-GST antibodies. When we used SPR to monitor the formation of complex of an anti-apoptotic protein

BAG-1 with the molecular chaperone Hsc70, the availability of one of the partners as a GST-fusion protein facilitated the binding analyses (12).

KEY RESEARCH ACCOMPLISHMENTS

- Crystallization of Rb "pocket domain."
- Purification of four GST-PR domain fusion proteins.
- Crystallization of wild-type PDFI-BF1 PR domain.
- Generation of 8 mutant PDF-BF1 PR domain proteins.

REPORTABLE OUTCOMES

None

CONCLUSIONS

The progress to date is on schedule testing the feasibility for crystallization and structure solution of the interesting PR domain of RIZ family proteins. Atomic models generated from the planned crystallographic studies will provide a critical insight into the structural and functional roles of these proteins in malignancy. Because RIZ is a newly characterized factor, the structural data generated will be novel in the search for a biochemical understanding of malignant transformation in breast cancer. RIZ or the interacting surface between Rb and RIZ may be innovative targets for drug regulation of cell cycling. Further, the new PR domain itself may be an entirely novel candidate for a drug since this motif is needed for tumor suppressor activity of RIZ. When this domain is missing, the propensity of development of tumors is greatly increased. It has been suggested that the PR domain may function in regulating chromatin-mediated gene expression. The molecular models that will ultimately be produced in this study will provide unparalleled molecular detail of the interacting proteins that can serve as the framework to understand the role of these tumor suppressors in breast cancer. In particular, the structures of the RIZ PR domain may suggest other functional roles for this interesting protein. The translational potential for this new regulator of tumorigenesis in breast cancer is real.

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APPENDICES

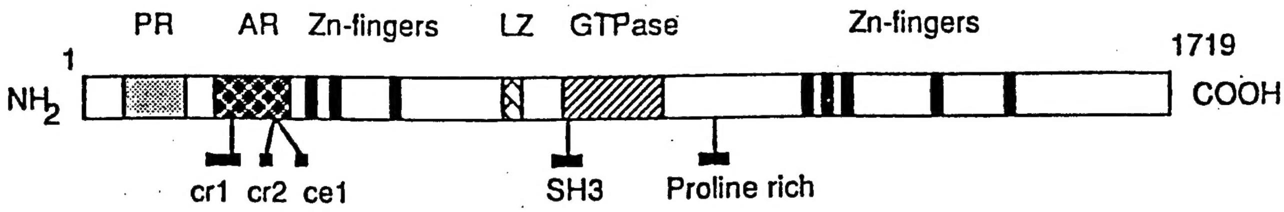

Figure 1. Schematic map of the human RIZ protein. The AR and PR domains are targeted in the proposed study.

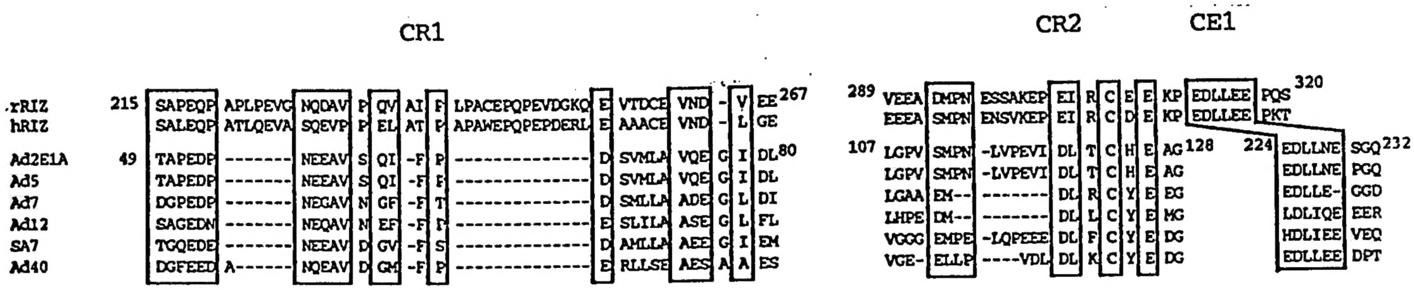

Figure 2. Similarities between RIZ AR domain and the conserved motifs in E1A. The rat ® and human (h) RIZ sequences are aligned by homology with the E1A protein sequences of various adenovirus strains. Identical or highly homologous residues are enclosed in boxes. Three segments of homology are shown: CR1 and CR2 as well as CE1 which is a region of antigenic homology.

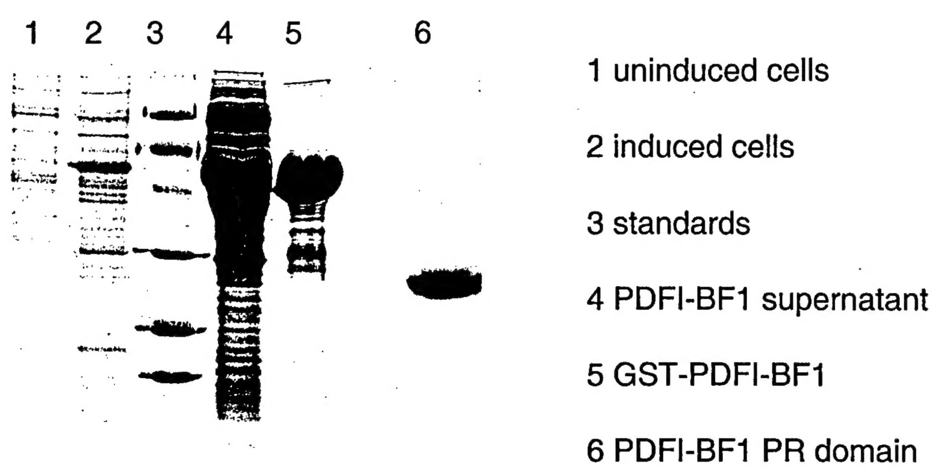

Figure 3: SDS-PAGE gel electrophoretic pattern monitoring the purification of the PR domain from PDFI-BF1. Molecular weight markers are included in lane three for comparison.

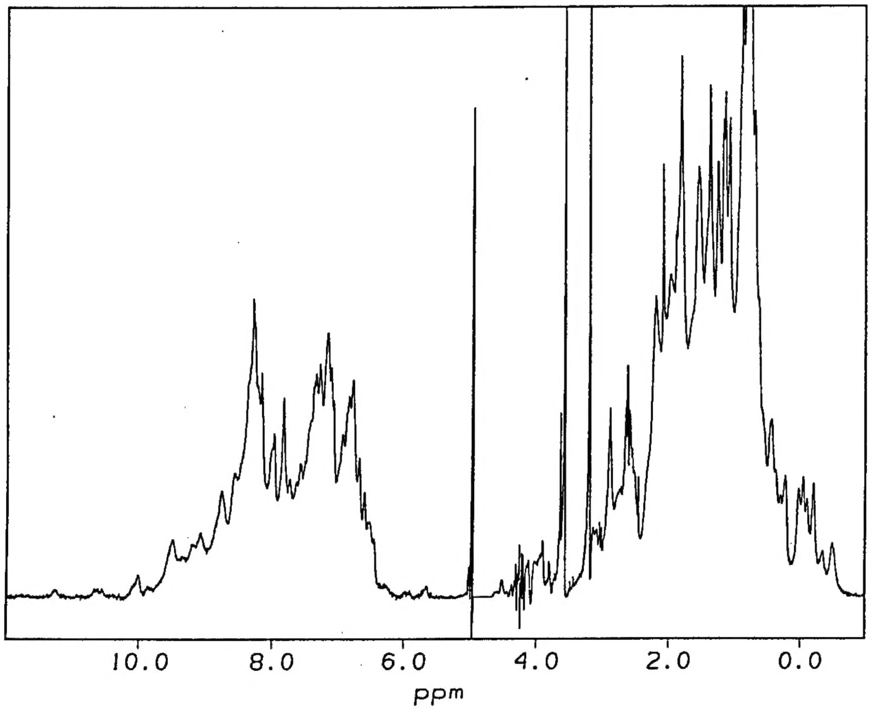

Figure 4: 1D ^{1}H NMR spectrum of BLPR #7 at 25° C in 10mM potassium phosphate buffer, 25mM KCl, 1mM EDTA, 1mM DTT, pH 6.8. Watergate was used for water suppression.